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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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VRIJE UNIVERSITEIT BRUSSEL
Pleinlaan 2
1050 Brussel
BELGIQUE

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Method of generating islet beta-cells from exocrine pancreatic cells

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METHOD OF GENERATING ISLET BETA-CELLS FROM EXOCRINE PANCREATIC CELLS

Background

Understanding the regulation of beta-cell neogenesis in the pancreas could lead to applications in the field of cell replacement or regeneration therapies for diabetes (1). For instance, islet transplantation can restore the functional beta-cell mass in diabetes patients (2), but it is seriously hampered by the shortage in donor tissue. This problem could be solved by finding ways of generating more islet cells from the available pancreatic tissue, by the process of neogenesis (3). Despite a lot of progress in the understanding of pancreas development during the last decade, the extracellular factors that specify islet cell differentiation in the embryo remain unknown (4). In adult mammals, the endocrine pancreas can expand or regenerate under certain experimental conditions, mainly as a result of islet cell neogenesis from progenitor cells (3). The exact nature of the latter remains elusive while duct cells, acinar cells, or intraislet cells having been suggested to have progenitor capacity (3-7). It is difficult to draw firm conclusions from whole pancreas studies both with respect to cell derivation and to the specific regulatory factors. Therefore, in vitro models are preferred to study islet neogenesis starting from defined cell preparations isolated from the pancreas. It has already been reported that additional islet cells could be generated from monolayer cultures of adult pancreatic tissue. In cultures derived from human pancreas and that were treated with KGF (keratinocyte growth factor) and nicotinamide, increases in insulin content were noted over a 3-4 week period. Also, cystic structures containing islet cells budded from the monolayer under influence of extracellular matrix (7). In another study, long-term cultures were obtained from diabetic mouse pancreas under glucose-free conditions, and these could be stimulated to generate islet-like structures in the presence of glucose (8).

Confirmation of these findings, in order to unravel the nature of the progenitor cells, of the regulatory factors, and to improve the efficacy of generating islet cells is still lacking.

We have proposed that differentiated exocrine cells can revert to a partially dedifferentiated state thereby re-acquiring embryonic plasticity (3,9-11). This indicated that exocrine cells, the great majority of cells in this organ, can be brought to transdifferentiate into endocrine cells under the appropriate conditions.

We have previously published that exocrine acinar cells can transdifferentiate into endocrine beta-cells (5) and there have been indications from in vivo studies for the existence of acinar-islet transitional cells (20,21). Since acinar cells can lose amylase and gain ductal characteristics (9,10), the appearance of transitional cells co-expressing ductal markers like cytokeratin and insulin (19) could also represent cells that were initially derived from acinar cells. It has been demonstrated that the amylase-secreting cell line AR42J, derived from an acinar tumor, can transdifferentiate into the beta-cell phenotype in vitro (22). The present invention reports the in vitro transdifferentiation of acinar cells into beta-cells in a primary culture model with a specific combination of growth factors.

EGF (Epidermal Growth Factor) and other EGF-family members have been implicated in the regulation of embryonic development as well as regeneration of the endocrine pancreas. EGF stimulates proliferation of the undifferentiated pancreatic precursor cells in vitro (23). In transgenic mice lacking functional EGF-receptors, islet morphogenesis is impaired and beta-cell differentiation is delayed (24). Betacellulin, a growth factor which also operates via the EGF-receptor, was found to promote islet regeneration in subtotally pancreatectomized rats (25) and in alloxan-diabetic mice (26). Glp-1, another factor that stimulates beta-cell neogenesis (27), has also been shown to transactivate the EGF-receptor (28). In combination with gastrin hormone, EGF was shown to stimulate beta-cell regeneration in streptozotocin-diabetic rats (29).

LIF (Leukemia Inhibitory Factor) is a pleiotropic cytokine for which a function in pancreatic development has so far not been described. It is a well-known regulator of stem cell proliferation and differentiation and is widely used to prevent differentiation of embryonic stem cells. Recently, it was reported to

stimulate the proliferation of multipotent adult progenitor cells in combination with EGF and PDGF (18).

Summary of the invention

The present invention relates to a method of generating *in vitro* insulin producing mammalian beta cells from dedifferentiated pancreatic cells. In one embodiments this is done by incubating said dedifferentiated pancreatic cells in a medium comprising a ligand of the EGF receptor, for example EGF or TGF-alpha. Optionally, the medium further comprises a ligand of the gp130 receptor such as LIF. As an alternative the medium optionally comprises bFGF. The dedifferentiated pancreatic cells used in this method are for example duct cells, acinar cells or islet cells. These dedifferentiated cells can be depleted of beta cells prior to the incubation into the above mentioned medium. The dedifferentiated pancreatic cells are mammalian cells obtained, for example, from rodents (rat, mouse), cattle, pigs and primates including humans.

In another aspect, the present invention relates to a population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the above described method. Such a population of insulin producing cells shows preferably an at least 2 fold increase in insulin secretion when exposed to glucose (e.g. 20 mM glucose for 4 hours). The population of cells can be further characterised by their immunoreactivity for markers such as C-peptide-I, Pdx-1 and Glut-2. They can also in addition be characterised by the presence of less than 10 % cytokeratin positive cells and/or less than 7 % binuclear cells.

The invention relates in a third aspect to a pharmaceutical composition comprising a cell population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the method of the present invention, further comprising at least one pharmaceutically acceptable carrier. A cell population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the method of the present invention can be used for the manufacture of a medicament for the treatment of diabetes type 1 or type 2. Thus, the invention also relates to a method for the treatment of diabetes comprising the step of administering the pharmaceutical composition of the present invention to an individual in need of it.

In another aspect the invention relates to the use of a ligand of the EGF receptor (e.g. EGF) or a ligand of the gp130 receptor (e.g. LIF) for the preparation of a medicament for the treatment of diabetes, in order to increase *in vivo* the amount of insulin secreting beta cell population.

In the present invention a reproducible primary culture model from adult rat pancreas was designed to induce islet beta-cell neogenesis in vitro. The present inventors have found that LIF can play a useful role in the development and regeneration of rat pancreas and islet tissue. The present invention discloses that EGF stimulates the derivation of insulin-expressing beta-cells from exocrine epithelial monolayers, e.g. in humans. In particular one aspect of the present invention is a combination of two factors, EGF and LIF to stimulate the derivation of insulin-expressing beta-cells from exocrine epithelial monolayers in rodents, especially rats.

As indicated above, application of islet transplantation as a treatment for diabetes is hampered by an inadequate supply of insulin-producing cells. In the present invention insulin-producing beta cells are generated from exocrine cells, which represent the great majority of cells in the pancreas, e.g. in humans and other mammals. In one embodiment, purified rat acinar cells were cultured in conditions leading to their de-differentiation into duct-like cells expressing cytokeratin-20. Monolayers of these cells were treated with a combination of epidermal growth factor (EGF) and leukemia inhibitory factor (LIF). In a period of three days, EGF plus LIF stimulated an 11-fold increase in the absolute number of insulin-positive cells, which couldn't be attributed to proliferation of contaminating beta-cells. A comparable increase could also be obtained from cultures that had been depleted of contaminating beta-cells by alloxan treatment. There was a similar increase in cellular insulin content of cultures with or without alloxan treatment. When EFG+LIF treated cells were exposed to 20 mM glucose over 4 hours, they showed a 4-fold increase in insulin secretion compared to basal glucose. Insulin-positive cells were immunoreactive for C-peptide-I, Pdx-1 and Glut-2, which are characteristics of mature beta-cells. Approximately 10% of insulin-positive cells were immunoreactive for cytokeratin-20 and 7 % were binuclear, which are both characteristics of the exocrine cells. Thus, exocrine cells can be directed to

differentiate into glucose-responsive beta-cells in vitro in the presence of EGF and LIF.

In yet another embodiment of the invention patients are treated with a ligand of a EGF receptor such as EGF and optionally bFGF or a ligand of the gp130 receptor such as LIF to stimulate expansion of the beta-cell mass present in the pancreas of an individual.

Brief description of the figures

Figure 1 shows an increase in DNA (A), % insulin-positive cells (B), beta-cell number (C), cellular insulin content (D) of cultures grown in the presence of EGF and LIF compared to cultures without these growth factors.

Detailed description of the invention

The present invention makes use of the exocrine fraction which is normally discarded after isolation of islets of Langerhans from the pancreas of humans and other mammals. We previously showed that these exocrine acinar cells change their phenotype profoundly within only 4 days of suspension culture (9,10). They lose amylase and other zymogens, and start to express the ductal marker CK20. In Matrigel matrix they form cystic structures resembling ducts, but when they remain in suspension culture they tend to dedifferentiate and express embryonic markers like the combination of Pdx-1 and Ptf1-p48 transcription factors (9) which is reminiscent of the protodifferentiated pancreatic progenitors (16), the neuroendocrine marker PGP9.5, and the CCKB gastrin receptor (9,10). In one embodiment of the present invention, such cells derived from rats were cultured as monolayers attached to plastic, in the presence of low serum-concentration (1% FBS) and were tested for the effect of a combination of EGF and LIF. This combination of growth factors, together with PDGF, was previously found to maintain the undifferentiated state of mesenchymal stem cells derived from adult marrow (18). In our model of dedifferentiated exocrine cells, this combination (EGF + LIF and optionally PDGF) was found, on the contrary, to stimulate cell proliferation as evidenced by a significant increase in total DNA and a larger area occupied by the monolayers. The combination of EGF and LIF, without PDGF also resulted in

an approximate six-fold increase in the percentage of insulin-positive cells, as compared to the initial cell number. Combined with the observed increase in DNA as a measure of cell number, this gives more than a tenfold increase in number of insulin-containing cells. The majority of these insulin-containing cells are classified as mature beta-cells since they expressed other phenotypic characteristics of beta-cells, such as the insulin-transactivating transcription factor Pdx-1, the beta-cell specific glucose transporter Glut-2, and the C-peptide-I component of unprocessed proinsulin. There were only very few BrdU-labeled beta-cells so that the observed increase in their number within a period of 3 days can be attributed to the differentiation of precursor cells, or neogenesis. There were two indications that the exocrine cells served as precursor cells of the newly formed beta-cells. First, CK20 immunoreactivity was noted in part of the insulin-positive cells. We have shown before that beta-cells containing CK20 are only found in the fetal (14) and neonatal pancreas (13), and in adult pancreas when neogenesis has been induced by duct-ligation (19). Thus, CK20 expression is a good indication for a transition from the CK20-positive exocrine cells to beta-cells. Second, binuclearity was noted in part of the insulin-positive cells. Binuclearity is a characteristic of acinar exocrine cells, and is not observed in normal rat beta-cells. This is another indication for a transition from exocrine cells to beta-cells.

In conclusion, the present invention includes a short-term culture model in which beta-cell neogenesis can be induced in certain animals such as rodents, especially rats by the combination of two soluble factors in the medium, namely EGF and LIF. This is the first in vitro study of acinoinsular transdifferentiation, documenting the phenotypic switch from normal exocrine to endocrine cells. For humans, LIF is optional. When applied to human cells, the present invention provides an important advancement in the treatment of diabetes by islet transplantation, by providing a way to overcome the problem of insufficient donor beta-cells.

The difference in behaviour of rodent (mouse, rat) and primate (human, monkey) cells towards LIF has been described for human ES cells and MAPC cells [18]. According to one embodiment human dedifferentiated pancreatic

cells are incubated in a composition comprising a ligand for the EGF receptor (for example EGF) and a ligand for the gp130 receptor (for example LIF).

In another embodiment of the invention human dedifferentiated pancreatic cells are incubated in a composition comprising a ligand for the EGF receptor (for example EGF, Transforming Growth Factor-alpha, Amphiregulin or Pox Virus Growth Factor) and a ligand for the bFGF receptor (e.g. basic FGF). Optionally, the composition for incubating human dedifferentiated pancreatic cells further comprises serum (e.g. FCS) and/or human dedifferentiated cells are grown on feeder layers.

Examples

Example 1: Experimental setup

Rats: Male adult Wistar rats (Janvier, Le Genest-St-Isle, France) weighing 250-300 g were used for the isolation of cells from the pancreas, as approved by the Ethical Committee of the Free University of Brussels.

Isolation of exocrine tissue: Pancreata were partially dissociated with collagenase and exocrine acini were purified by centrifugal elutriation as published before (9). The resulting exocrine fraction which was used in this study was composed of at least 90% acinar cells as judged from amylase immunostaining.

Culture procedure: Exocrine cells were pre-cultured for 4 days in suspension culture in RPMI-1640 Glutamax-I medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (Gibco BRL, Paisley, Scotland). Cellular aggregates were sedimented at unit gravity and medium was replaced daily during this preculture period. On the fourth day after isolation, the cell aggregates were centrifuged and after determination of the pellet volume, it was resuspended in culture medium and cells were distributed in 1000 µl-aliquots over 24-well plates (Falcon, BD Biosciences, Erembodegem, Belgium). This procedure was standardized as to obtain approximately 75 ng DNA per well. For some experiments, the cells were treated with 10 mM alloxan for 2 hours prior to plating. After overnight culture, nonadherent cells were washed off and then either control medium or growth factor-containing medium was added to the wells. Control medium consisted of RPMI-1640 medium supplemented with

1% FBS. Growth factor-medium consisted of control medium supplemented with 50 ng/ml human recombinant epidermal growth factor (EGF) (Sigma, St. Louis, MO) and 40 ng/ml recombinant mouse leukemia inhibitory factor (LIF) (Sigma). Cell monolayers were analysed after a culture period of 3 days in the latter media.

Immunocytochemistry and DNA measurement: The amount of cells per well was estimated by measuring DNA with a fluorimetric assay based on the binding of Hoechst 33258 dye (12). At least 6 wells were used per experimental condition, so that triplicate cultures could be used for DNA-extraction and, in parallel, for immunocytochemical analysis of cell composition. Immunocytochemical staining of the monolayers was performed directly in the 24-well plates. For this purpose, the cell monolayers were fixed for 10 min with 4% buffered formaldehyde followed by 20 min methanol (-20°C) for cell permeabilization. For single stainings of only one antigen, we used the streptavidin-biotin peroxidase method (13,14). For double stainings, we used the indirect method with FITC- and TRITC-labeled secondary antibodies (Jackson Immunoresearch, West Grove, PA). Primary antibodies used in this study are polyclonal anti-insulin (gift from C. Van Schravendijk, VUB, Brussels) (13,14), polyclonal anti-rat C-peptide-I (O.D. Madsen, Hagedorn Research Institute, Gentofte, Denmark)(15), polyclonal anti-Pdx1 (O.D. Madsen)(9), polyclonal anti-Glut-2 (Wak-Chemie, Bad Soden, Germany), monoclonal anti-cytokeratin-20 (CK20) (Novocastra, Newcastle-upon-Tyne, UK)(13,14), polyclonal anti-alpha-amylase (Sigma), and mouse monoclonal anti-BrdU (ICN, Irvine, CA, USA). To assess the incorporation of 5'-bromodeoxyuridine (BrdU) by proliferating cells, we added 50 μM BrdU (Sigma) to the culture medium one hour before fixation (10).

Morphometry: We used computer-assisted morphometry (11) to measure the area of monolayers in 24-well plates.

Insulin measurements: Cellular insulin content and insulin released in the medium were measured by radio-immunoassay (16). To study glucose-stimulated insulin release, insulin in the culture medium was measured after a 4 hour incubation in basal medium containing 2.5 mM glucose, followed by a 4

hour incubation in 20 mM glucose (serum-and glutamine-free HAM-10 medium, Gibco).

Statistics: We used a two-tailed, paired Student t-test and considered statistical significance at a confidence interval <0.05. Mean values are given ± SEM. Experiments were repeated at least 5 times independently with each cell preparation being a pool from a total of 5 rats.

Example 2: Generation and composition of exocrine cell monolayers

We isolated and pre-cultured exocrine acinar cell aggregates as described earlier (9,10) and subsequently allowed these cells to form monolayers on plastic. More than 90% of the cells were immunoreactive for the ductal marker CK20 and had lost the amylase marker of acinar cells. Of these cells, 79.0 ± 0.4% (n=7) were binuclear, a characteristic of acinar cells. The cultures were initially contaminated with 3.7 ± 0.46 % (n=7) insulin-positive cells.

Example 3: Effect of growth factors on cell number and beta-cell frequency

When the monolayer cultures obtained in example 2 were treated with EGF and LIF for 3 days under the conditions described in example 1, we noted a significant increase in DNA content compared to the start of the culture and compared to cultures devoid of both growth factors (FIG. 1A). The total surface covered with cells showed a comparable increase.

Treatment with EGF plus LIF also induced a significant increase in the frequency of insulin-positive cells, expressed as the percentage of all cells (FIG. 1B). When multiplying the frequency of insulin-positive cells with total DNA-content to have a measure of the absolute beta-cell number, we observed an 11-fold increase in beta-cell number in the presence of EGF plus LIF over the 3-day culture period (FIG. 1C). Analysis of total cellular insulin content by radioimmunoassay showed a 6-fold increase in EGF + LIF treated cultures compared to control cultures (FIG. 1D).

In the presence of EGF or LIF alone there was also a significant increase in beta-cell number, respectively 6- and 5-fold compared to the start material,

but these increases were significantly less than in the presence of both factors together.

Example 4: Effect of growth factors on cell number and beta-cell frequency on Alloxan pre-treated cultures

In a set of parallel experiments, the exocrine acinar cell preparations were pre-treated with alloxan to destroy contaminating beta-cells prior to monolayer formation and stimulation with growth factors. These monolayers contained less than 0.3% insulin-positive cells. After 3 days of culture in the presence of EGF and LIF we found 3.8 ± 0.31% insulin-positive cells. This re-appearance of insulin-positive cells was not observed in the absence of growth factors nor in the presence of EGF alone. This is different from what we observed without alloxan treatment where EGF alone did induce an increase in insulin-positive cell number. This discrepancy could be explained by a paracrine effect from beta-cells or other alloxan-sensitive cells in the culture mimicking the effect of LIF. This could also explain the observation that in the absence of added growth factors, there was a 1.8-fold increase in insulin-positive cell number compared to the start (Fig. 1C). We also noted that the size of the monolayers from alloxan-treated cells were smaller than from non-alloxan treated cells, indicating that alloxan also caused some damage to the non-beta cells.

Example 5: Phenotypic analysis

Analysis by double immunofluorescence staining showed that most of these insulin-positive cells also expressed other beta-cell markers, including proinsulin C-peptide-I, the transcription factor Pdx-1, and the glucose transporter Glut-2. Immunoreactivity for C-peptide excludes the possibility of insulin uptake from the medium as an explanation for the increased frequency of insulin-immunoreactive cells.

Most of the insulin-positive cells were negative for CK20. However, in the EGF+LIF treated cultures, we noted that approximately 10% of the insulin-positive cells were immunoreactive for CK20, with a lower staining intensity compared to insulin-negative cells. Co-expression of insulin and CK20 was

rarely observed in control cultures where no growth factors were added and it was not seen in the starting material. This co-expression suggests a phenotypic transition from exocrine to insulin-positive cells. Strikingly, we observed insulin and CK20-double-positive cells that were binuclear. Binuclear insulin-positive cells were rarely (<1%) seen in control cultures and were absent at the start, but they were frequently seen in EGF+LIF-treated cultures where they accounted for 6.2 ± 0.47 % (n= 7) of all insulin-positive cells. In these exocrine cell-derived monolayers, nearly 80% of the cells were binuclear. Since binuclearity is a characteristic of the majority of acinar exocrine cells (9), these observations are another indication for a transition from exocrine cells to insulin-positive cells. Analysis of BrdU-incorporation in insulin-positive cells showed that less than 0.1% of insulin-positive cells were BrdU-labelled. This makes it highly unlikely that binuclearity in these cells would have resulted from arrested nuclear division. Interestingly, in EGF+LIF-treated cultures we counted a small but statistically significant decrease in the frequency of binucleated CK20-positive cells as compared to control cultures, namely from 79.0 ± 0.40% to 69.4 ± 0.65% (n=7). This suggests that the growth factors stimulated cytokinesis in part of the binuclear exocrine cells.

Example 6: Insulin secretion

Insulin secretion studies were done over 4 h in culture. Compared to their basal secretion in 2.5 mM glucose, stimulation by 20 mM glucose gave a 4-fold increase in secreted insulin, namely from 2.3 to 9.5 ng/ml.

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Claims

1. A method of generating *in vitro* insulin producing mammalian beta cells from dedifferentiated pancreatic cells comprising the step of : -incubating said dedifferentiated pancreatic cells in a medium comprising a ligand of the EGF receptor.

2. The method according to claim 1 wherein the ligand of the EGF receptor is EGF.

3. The method according to claim 1 or 2 wherein the medium further comprises a ligand of the gp130 receptor.

4. The method according to any of claims 1 to 3 wherein the ligand of the gp130 receptor is LIF.

5. The method according to any of claims 1 to 2 wherein the medium further comprises bFGF.

6. The method according to any of claims 1 to 5 wherein the dedifferentiated cells are duct cells.

7. The method according to any of claims 1 to 6 wherein the dedifferentiated pancreatic cells are depleted of beta cells.

8. The method according to claim 1-7 wherein the dedifferentiated pancreatic cells are selected from the group of duct cells, acinar cells and islet cells.

9. The method according to any of claims 1-7 wherein the mammalian cells are human cells.

10. A population of insulin producing cells obtainable by the method of any of claims 1 to 9.

11. The population of insulin producing cells according to claim 10 wherein said cell population shows a more than 2 fold increase in insulin secretion when exposed to 20 mM glucose for 4 hours.

12. The population of insulin producing cells according to claim 10 or 11 wherein said cell population is immunoreactive for C-peptide-I, Pdx-1 and Glut-2.

13. The population of insulin producing cells according to any of claims 10 to 12 wherein said population comprises less than 10 % cytokeratin positive cells and/or less than 7 % binuclear cells.

14. A pharmaceutical composition comprising a cell population according to any of claims 10-13 and at least one pharmaceutically acceptable carrier.

15. Use of a cell population according to any of claims 10-13 of for the manufacture of a medicament for the treatment of diabetes type 1 or type 2.

16. A method for the treatment of diabetes comprising the step of administering the pharmaceutical composition of claim 7 to an individual in need of it.

17. Use of a ligand of the EGF receptor an optionally ligand of the gp130 receptor for the preparation of a medicament for the treatment of diabetes.

18. Use according to claim 17 wherein the ligand of the EGF receptor is EGF and the ligand of the gp130 receptor is LIF.

Abstract

The present invention discloses an *in vitro* method wherein mammalian beta-cell neogenesis can be induced in a medium comprising EGF and LIF. Human insulin secereting cells, obtainable by this method, provide a means for the treatment of diabetes by islet transplantation.

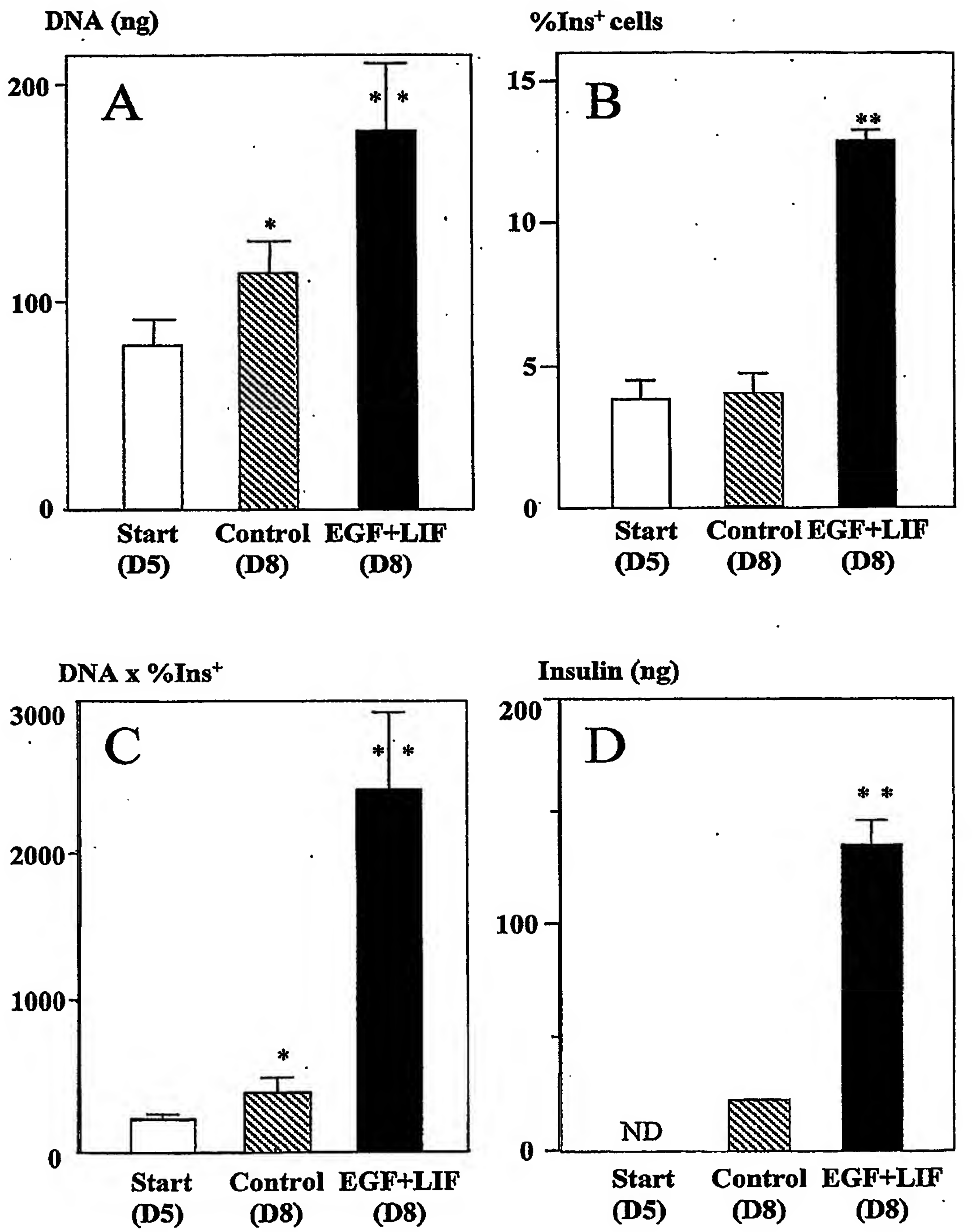
DNA (ng)
200
100
0
A
Start (D5)
Control (D8)
EGF+LIF (D8)
%Ins+ cells
15
10
5
0
B
Start (D5)
Control (D8)
EGF+LIF (D8)
DNA x %Ins+
3000
2000
1000
0
C
Start (D5)
Control (D8)
EGF+LIF (D8)
Insulin (ng)
200
100
0
D
ND
Start (D5)
Control (D8)
EGF+LIF (D8)

Fig. 1

PCT/BE2004/000089

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE2004/000089

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2002/155598 A1 (KERR-CONTE JULIE ET AL) 24 October 2002 (2002-10-24) "Process for obtaining mammalian insulin secreting cells in vitro and their uses" abstract page 3, left-hand column, paragraph 50 - right-hand column, paragraph 62 -----	1-21,24, 28-40, 42-44

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 2004	18. 02. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niebuhr-Ebel, K

INTERNATIONAL SEARCH REPORT

International Application No
PCT/BE2004/000089

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01/32839 A (UNIV MCGILL ; ROSENBERG LAWRENCE (CA)) 10 May 2001 (2001-05-10) "Medium for preparing dedifferentiated cells" page 8, line 15 - line 21 page 21, line 21 - line 34 page 22, line 26 - page 23, line 34 page 26, line 2 page 34, line 25 - page 35, line 25 -----	1-21,24, 28-40, 42-44
A	VITI JANE ET AL: "Epidermal growth factor receptors control competence to interpret leukemia inhibitory factor as an astrocyte inducer in developing cortex." THE JOURNAL OF NEUROSCIENCE : THE OFFICIAL JOURNAL OF THE SOCIETY FOR NEUROSCIENCE. 15 APR 2003, vol. 23, no. 8, 15 April 2003 (2003-04-15), pages 3385-3393, XP002299605 ISSN: 1529-2401 cited in the application the whole document -----	1-21,24, 28-40, 42-44

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BE2004/000089

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 35 (partially)
because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-21, 24, 28-40, 42-44

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-21, 24, 28-40, 42-44

   Method of generating insulin producing beta cells from a population comprising dedifferentiated exocrine pancreatic cells by adding ligands of the gp130 receptor and/or the EGF receptor, cell populations obtainable by said method, use of said method and cells.

   ---

2. claims: 22, 23, 25-27, 41

   Cell population having features of differentiated and undifferentiated beta cells in the same individual cell, and a method for determining the degree of redifferentiation using markers for such cells.

INTERNATIONAL SEARCH REPORT

In[redacted]mation on patent family members

Internati[redacted]Application No
PCT/BE2004/000089

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 2002155598	A1	24-10-2002	FR	2814752	A1	05-04-2002
			AU	9197101	A	15-04-2002
			WO	0229010	A2	11-04-2002
WO 0132839	A	10-05-2001	AU	1123401	A	14-05-2001
			WO	0132839	A2	10-05-2001
			CA	2388208	A1	10-05-2001
			EP	1224263	A2	24-07-2002
			JP	2003513624	T	15-04-2003
			MX	PA02004268	A	28-01-2003
			US	2004018623	A1	29-01-2004
			US	2004033599	A1	19-02-2004

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(71) Applicant (*for all designated States except US*): VRIJE UNIVERSITEIT BRUSSEL [BE/BE]; Pleinlaan 2, B-1050 Brussel (BE).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): BOUWENS, Luc [BE/BE]; Duvelsborreweg 1, B-1653 Dworp (BE). BAEYENS, Luc [BE/BE]; Stationstraat 22, B-1730 Asse (BE).

(74) Agents: BIRD, William, E. et al.; Bird Goën & Co, Klein Dalenstraat 42A, B-3020 Winksele (BE).

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Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

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(54) **Title:** METHOD OF GENERATING ISLET BETA-CELLS FROM EXOCRINE PANCREATIC CELLS

(57) **Abstract:** The present invention discloses an *in vitro* method wherein mammalian beta-cell differentiation can be induced in dedifferentiated exocrine pancreatic cells in a medium comprising ligands of the EGF receptor and the GP130 receptor, such as EGF and LIF. Insulin secreting cells, obtainable by this method, provide a means for the treatment of diabetes by islet transplantation.

WO 2004/113512 A2

METHOD OF GENERATING ISLET BETA-CELLS FROM EXOCRINE PANCREATIC CELLS

Field of the invention

The present invention relates to methods of differentiating pancreatic cells. The present invention further relates to insulin secreting cell populations for the treatment of diabetes.

Background of the invention

Understanding the regulation of beta-cell neogenesis in the pancreas could lead to applications in the field of cell replacement or regeneration therapies for diabetes [Yamaoka T. in *Biochem Biophys Res Commun.* (2002) **296**, 1039-43]. For instance, islet transplantation can restore the functional beta-cell mass in diabetes patients [Shapiro *et al.* in *N Engl J Med.* (2000) **343**, 230-238], but it is seriously hampered by the shortage in donor tissue. This problem could be solved by finding ways of generating more islet cells from the available pancreatic tissue, by the process of neogenesis [Bouwens & Kloppel in *Virchows Arch.* (1996) **427**, 553-560]. Despite a lot of progress in the understanding of pancreas development during the last decade, the extracellular factors that specify islet cell differentiation in the embryo remain unknown [Edlund in *Nat Rev Genet.* (2002) **3**, 524-532]. In adult mammals, the endocrine pancreas can expand or regenerate under certain experimental conditions, mainly as a result of islet cell neogenesis from progenitor cells [Bouwens & Kloppel *cited supra*]. The exact nature of the latter remains elusive while duct cells, acinar cells, or intraislet cells have been suggested bear progenitor capacity [Bouwens & Kloppel in *Virchows Arch.* (1996) **427**, 553-560; Edlund in *Nat Rev Genet.* (2002) **3**, 524-532; Bouwens in *Microscopy Research and Technique*.(1998) **43,** 332-336; Guz *et al.* in *Endocrinology.* (2001) **142**, 4956-4968; Bonner-Weir *et al.* in *Proc Natl Acad Sci USA.* (2000) **97,** 7999-8004; Ramiya VK *et al. Nat Med.* (2000) **6**, 278-282].

It is difficult to draw firm conclusions from whole pancreas studies both with respect to cell derivation and to the specific regulatory factors. Therefore, in

vitro models are preferred to study islet neogenesis starting from defined cell preparations isolated from the pancreas. Very few *in vitro* studies have been able to demonstrate the feasibility of inducing islet neogenesis from adult tissue. It has already been reported that additional islet cells could be generated from monolayer cultures of adult pancreatic tissue. Confirmation of these findings, in order to unravel the nature of the progenitor cells, of the regulatory factors, and to improve the efficacy of generating islet cells is still lacking.

Human pancreatic derived cell cultures that were treated with KGF (keratinocyte growth factor) and nicotinamide, resulted in increases in insulin content after 3 to 4 weeks. Also, cystic structures containing islet cells budded from the monolayer under influence of extracellular matrix [Bonner-Weir et al. *cited supra*]. The precursors responsible for this neogenesis were characterised as cells expressing the ductal marker cytokeratin-19 [Gao et al.*cited supra*]. In another study, long-term cultures were obtained from diabetic NOD mouse pancreas under glucose-free conditions, and these could be stimulated to generate islet-like structures in the presence of glucose [Ramiya VK *et al.* in *Nat Med.* (2000) **6**, 278-282]. These cells did not reach functional maturity *in vitro*. It is at present unclear whether the latter observations may have been due to "passenger" stem cells derived from the blood circulation, which have been discovered recently in NOD mice [Kodama et al. in *Science* (2003) **302**, 1223-1227].

Differentiated exocrine cells can revert to a partially dedifferentiated state thereby re-acquiring embryonic plasticity [Bouwens & Kloppel in *Virchows Arch.* (1996) **427**, 553-560; Rooman *et al.* in *Diabetologia* **43**, 907-914 (2000); Rooman *et al.* in *Gastroenterology* **121**, 940-949 (2001); Rooman *et al.* in *Diabetes* **51**, 686-690, (2002)]. This indicated that exocrine cells, the great majority of cells in this organ, can be brought to transdifferentiate into endocrine cells under the appropriate conditions.

Exocrine acinar cells can transdifferentiate into endocrine beta-cells [Bouwens in *Microscopy Research and Technique* (1998) **43,** 332-336] and there have been indications from *in vivo* studies for the existence of acinar-islet transitional cells [Gu *et al.* in *Pancreas* (1997) **15**, 246-2501; Bertelli E,

Bendayan M in *Am J Physiol* (1997) **273**, C1641-C1649]. Since acinar cells can lose amylase and gain ductal characteristics [Rooman et al.(2000) & (2001) *cited supra*] the appearance of transitional cells co-expressing ductal markers like cytokeratin and insulin [Wang *et al.* in *Diabetologia.* (1995) **38**, 1405-1411] could also represent cells that were initially derived from acinar cells. It has been demonstrated that the amylase-secreting cell line AR42J, derived from an acinar tumor, can transdifferentiate into the beta-cell phenotype *in vitro* [Mashima *et al.* in *J Clin Invest.* (1996) **97**, 1647-1654]. The present invention reports the *in vitro* transdifferentiation of acinar cells into beta-cells in a primary culture model with a specific combination of growth factors.

LIF (Leukemia Inhibitory Factor) is a pleiotropic cytokine for which a function in pancreatic development has so far not been described. It is a well-known regulator of stem cell proliferation and differentiation and is widely used to prevent differentiation of embryonic stem cells. Recently, it was reported to stimulate the proliferation of multipotent adult progenitor cells (without differentiation of the cells) in combination with EGF and PDGF [Jiang *et al.* in *Nature* (2002) **418**, 41-49].

EGF (Epidermal Growth Factor) and other EGF-family members have been implicated in the regulation of embryonic development as well as regeneration of the endocrine pancreas. EGF stimulates proliferation of the undifferentiated pancreatic precursor cells *in vitro* [Cras-Meneur *et al.* in *Diabetes.* (2001) **50**, 1571-1579]. In transgenic mice lacking functional EGF-receptors, islet morphogenesis is impaired and beta-cell differentiation is delayed [Miettinen et al. in *Development.* (2000) **127**, 2617-2627]. Betacellulin, a growth factor which also operates via the EGF-receptor, was found to promote islet regeneration in subtotally pancreatectomized rats (25li) and in alloxan-diabetic mice [Yamamoto *et al.* in *Diabetes.* (2000) **49**, 2021-2027]. Glp-1, another factor that stimulates beta-cell neogenesis [Drucker in *Mol Endocrinol.* (2003) **17**,161-171] has also been shown to transactivate the EGF-receptor [Buteau *et al.* in *Diabetes.* (2003) **52**, 124-132]. In combination with gastrin hormone, EGF was shown to stimulate beta-cell regeneration in

streptozotocin-diabetic rats [Brand *et al.* in *Pharmacol Toxicol.* (2002) **91**, 414-420].

LIF and EGF have been reported to act synergistically as signals that regulate the differentiation of neurons and glial cells in embryos [Viti et al. in *J. Neurosci.* (2003) **15**, 3385-3393]. In astrocyte progenitors EGF increases the competence to interpret LIF as an astrocyte-inducing signal via increased STAT3 phosphorylation. LIF is also considered a key signal for injury-induced neurogenesis in the adult [Bauer et al. in *J. Neurosci.* (2003) **23**, 1792-1803].

Summary of the invention

According to one aspect the present invention relates to an *in vitro* method of generating insulin producing beta cells from a population comprising or consisting of dedifferentiated exocrine pancreatic cells of a first mammal, said method comprising the steps of: a) providing said population of dedifferentiated exocrine pancreatic cells in a culture medium, b) adding one or more ligands of the gp130 receptor of a second mammal and/or adding ligands of the EGF receptor of a third mammal to said culture medium, c) incubating said dedifferentiated exocrine pancreatic cells in said culture medium comprising said one or more ligands of the gp130 receptor and/or said one or more ligands of the EGF receptor.

In one embodiment the method is performed by adding in step b) one or more ligands of the gp130 receptor of a second mammal without adding one ore more ligands of the EGF receptor of a third mammal to said culture medium, In a particular embodiment the method is performed by adding in step b) both one or more ligands of the gp130 receptor of a second mammal as well as adding one or more ligands of the EGF receptor of a third mammal to said culture medium, According to one embodiment, the ligand of said gp130 receptor is LIF, such as human or humanised LIF. According to one embodiment LIF is added to the culture medium in a concentration between 10 and 100 ng/ml, or between 10 and 25 ng/ ml or between 100 and 500 ng/ ml. According to one embodiment the ligand of said EGF receptor is a human or humanised ligand of said EGF receptor. According to another embodiment the

ligand of said EGF receptor is EGF such as human or humanised EGF. According to one embodiment EGF is added to the culture medium in a concentration between 10 and 100 ng/ml or between 10 and 25 ng/ ml or between 100 and 500 ng/ ml. Generally, the one or more ligands of the gp130 receptor and/or one or more of the ligands of the EGF receptor are added to the culture medium in a concentration between 1 and 10 000 ng/ml. According to a particular embodiment, the method further comprises the step of adding bFGF (basic fibroblast growth factors) to said culture medium during step b. According to a particular embodiment the medium is free from KGF (keratinocyte growth factor) or a gastrin/CCK receptor ligand. In particular embodiments the incubation step in the present method is performed during 7, 6, 5 or even less than 5 days namely 4 or 3 days. The population of dedifferentiated exocrine pancreatic cells which can be used for the method of the invention is according to one embodiment selected from the group consisting of duct cells, acinar cells and islet cells. Also mixtures of these types cells, or also cell populations, comprising a certain ratio of one or more of the cells types consisting of the group consisting of duct cells, acinar cells and islet cells can be used in the present invention. According to another embodiment, an additional step prior to step a) is performed wherein beta cells are depleted. According to another embodiment, in order to reduce the growth of fibroblast cells, dedifferentiation and redifferentiation is performed in the presence of genitimycine. Cells which can be used according to the methods of the present invention are all types of mammalian cells including rodent, porcine, monkey and human cells. In a particular embodiment, the mammalian cells are rat cells, the one or more ligands of said EGF receptor comprise human EGF the one or more ligands of gp130 receptor comprise murine LIF.

Another aspect of the invention relates to a population of mammalian pancreatic cells comprising mammalian insulin producing beta cells obtainable by any of the here above describes embodiments of the method of the present invention. In one embodiment, this population of mammalian pancreatic cells comprises from about 5 to about 15 percent of insulin-positive cells. In another embodiment, this population of mammalian pancreatic cells, after exposure to

conditions such as to 20 mM glucose for 4 hours at 37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum shows a more than 2 fold increase in insulin secretion when compared to the insulin secretion prior to said exposure to glucose.

In another embodiment, this population of mammalian pancreatic cells are able to provide an insulin secretion of at least 10 ng/ml after exposure of said population to conditions such as 20 mM glucose for 4 hours at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine.

Another aspect of the invention relates to a population of cells comprising mammalian insulin producing beta cells wherein said cell population comprises cells having at least one feature of a differentiated beta cell and at least one feature of an undifferentiated beta cell in the same individual cell. A feature of a differentiated beta cell can be for example insulin secretion and a feature of an undifferentiated beta cell can be CK20 expression and/or binuclearity. The present invention also relates to such a population of mammalian pancreatic cells which is obtainable by any of the embodiments of the above described redifferentation method.

Another aspect of the present invention relates to a population of mammalian pancreatic cells comprising mammalian insulin secreting beta cells wherein said cell population comprises a first subpopulation of cells having markers of undifferentiated or dedifferentiated cells and comprises a second subpopulation of cells having markers of differentiated cells. Markers of differentiated cells are for example C-peptide-I, Pdx-1, Glut-2 or insulin. Markers of dedifferentiated or undifferentiated cells are for example cytokeratin 7, cytokeratin 19, cytokeratin 20, CCKB receptor for gastrin, PGP9.5 or notch-1 receptor. The present invention also relates to such a population of mammalian pancreatic cells which is obtainable by any of the embodiments of the above described redifferentation method.

In another aspect, the present invention relates to a pharmaceutical composition comprising a therapeutically active amount of a mammalian pancreatic cell population comprising redifferentiated cells which are obtainable by the method of the present invention.

In another aspect, the present invention relates the use of a mammalian pancreatic cell population comprising redifferentiated cells which are obtainable by the method of the present invention for the manufacture of a medicament. In a particular embodiment, the medicament is used for the treatment of diabetes type 1 or type 2.

In yet another aspect, the present invention relates to a method for the treatment of diabetes type 1 or type 2 comprising the step of administering an effective amount of the pharmaceutical composition comprising a therapeutically active amount of a mammalian pancreatic cell population comprising redifferentiated cells which are obtainable by the cultivation methods of the present invention

In yet another aspect, the present invention relates to the use of a combination of a human or humanised ligand of a EGF receptor, and a human or humanised ligand of the gp130 receptor for the preparation of a medicament. In one embodiment the medicament is used for the treatment of diabetes type 1 or type 2. In another embodiment the human or humanised ligand of a EGF receptor is human EGF and the human or humanised ligand of the human gp130 receptor is human LIF.

In yet another aspect the present invention relates to the use of a human or humanised ligand of the gp130 receptor for the preparation of a medicament for the treatment of diabetes type 1 or type 2. In one embodiment the human or humanised ligand of the gp130 receptor is LIF.

In yet another embodiment the invention relates to an *in vitro* method for determining the degree of redifferentiation of dedifferentiated mammalian pancreatic cells comprising the steps of determining one or more parameters selected from the group consisting of a) The presence of CK20, CK7 or CK 19, b) the ocurrence of binucleated cells, c) the presence of insulin positive cells, d) the presence of C-peptide, Pdx-1 and Glut-2, e) the presence of gastrin CCKB receptor, PGP9.5 and notch-1 receptor on said mammalian pancreatic cells. The invention also relates to a population of mammalian pancreatic cells being identifiably by this *in vitro* method for determining the degree of redifferentiation of dedifferentiated

The present invention relates to a method of generating *in vitro* insulin producing mammalian beta cells from dedifferentiated pancreatic cells. In one embodiment this is done by incubating said dedifferentiated pancreatic cells in a medium comprising a ligand of the EGF receptor, for example EGF or TGF-alpha. Optionally, the medium further comprises a ligand of the gp130 receptor such as LIF. As an alternative the medium optionally comprises bFGF. The dedifferentiated pancreatic cells used in this method are for example duct cells, acinar cells or islet cells. These dedifferentiated cells can be depleted from beta cells prior to the incubation into this medium. The dedifferentiated pancreatic cells are mammalian cells obtained, for example, from rodents (rat, mouse), cattle, pigs and primates including humans.

In another aspect, the present invention relates to a population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the above described method. Such a population of insulin producing cells shows preferably an at least 2 fold increase in insulin secretion when exposed to glucose (e.g. 20 mM glucose for 4 hours). The population of cells can be further characterised by their immunoreactivity for markers such as C-peptide-I, Pdx-1 and Glut-2. They can also in addition be characterised by the presence of less than 10 % cytokeratin positive cells and/or less than 7 % binuclear cells.

The invention relates in a further aspect to a pharmaceutical composition comprising a cell population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the method of the present invention, further comprising at least one pharmaceutically acceptable carrier. A cell population of insulin producing cells obtainable from dedifferentiated pancreatic cells by the method of the present invention can be used for the manufacture of a medicament for the treatment of diabetes type 1 or type 2. Thus, the invention also relates to a method for the treatment of diabetes comprising the step of administering the pharmaceutical composition of the present invention to an individual in need of it.

In another aspect, the invention relates to the use of a ligand of the EGF receptor (e.g. EGF) or a ligand of the gp130 receptor (e.g. LIF) for the

preparation of a medicament for the treatment of diabetes, in order to increase *in vivo* the amount of insulin secreting beta cell population.

As indicated above, application of islet transplantation as a treatment for diabetes is hampered by an inadequate supply of insulin-producing cells. In the present invention insulin-producing beta cells are generated from exocrine cells, which represent the great majority of cells in the pancreas, e.g. in humans and other mammals.

The present invention provides a method wherein beta-cell neogenesis can be induced from exocrine cells by the combination of two soluble factors in the medium, namely EGF and LIF. The invention provides an important advancement in the treatment of diabetes by islet transplantation, by providing a way to overcome the problem of insufficient donor beta-cells.

When applied to human cells, the present invention provides an important advancement in the treatment of diabetes by islet transplantation, by providing a way to overcome the problem of insufficient donor beta-cells.

Brief description of the figures

Figure 1 shows, according to an embodiment of the invention, an increase in DNA (A), % insulin-positive cells (B), beta-cell number (C), cellular insulin content (D) of cultures grown in the presence of EGF and LIF compared to cultures without these growth factors (* = $p<0.05$; ** = $p<0.01$). Cells are kept for 5 days in the medium (left) prior to a three day period without (middle) or with EGF+LIF (right).

Figure 2 shows, according to an embodiment of the invention, the increase of insulin-positive beta cells in control medium versus with both EGF and LIF treated cultures, after pretreatment with alloxan (A) ($n = 4$), number of BrdU-labeled insulin positive beta-cells in the presence of EGF and LIF during the pulse and the chase period (B) ($n = 4$). (** = $p<0.01$).

Figure 3 shows, according to an embodiment of the invention, an in vivo diabetic mouse model using EGF+LIF redifferentiated cells of the present invention.

Detailed description of the invention

"added", **"adding"** or **"addition"** in the present invention refers to compounds EGF receptor and Gp130 receptor ligands, such as LIF and EGF, which are supplemented separately to the medium. It does not refer to unknown levels of compounds which are present in the medium due to secretion by the cells. It also does not refer to low amounts of compounds which are present in serum which is added to a basal growth medium. Concentrations of added compounds in the medium are in the ng/ml range and may vary from about 1, 10, 25, 50, 100, 250, 500, up to 1000 ng/ml. In a specific embodiment the concentration of added compounds for each compound separately varies between 10 and 100 ng/ml. In another specific embodiment the concentration of added compounds for each compound separately varies between 20 and 100 ng/ml.

"Dedifferentiated exocrine pancreatic cells" refers to those cells which are to a lesser or greater extent dedifferentiated and have re-acquired embryonic plasticity. Typical features of differentiated cells which have been lost by the differentiated cells are the expression of amylase and other zymogens, such as pancreatic trypsinogen, trypsin and lipase, the insulin-transactivating transcription factor Pdx-1, the beta-cell specific glucose transporter Glut-2, and the C-peptide-I component of unprocessed proinsulin. Typical features of the embryonic plasticity which have been acquired by the dedifferentiated cells are the expression of cytokeratins 7, 19 and/or 20. In addition there can be expression of the cholecystokinin B CCKB-receptors for gastrin, the neuroendocrine markers PGP9.5 (neuron-specific ubiquitin c-terminal hydrolase) and the Notch-1 receptor. Typical pancreatic cell types which can be dedifferentiated according to the present invention are acinar cells, duct cells and non-endocrine islet cells.

"Beta cells" are generally known as specialized cells found in clusters (islets) in the pancreas. Beta cells regulate glucose levels in the bloodstream by making insulin, monitoring glucose levels, and secreting insulin in response to elevated glucose levels. Together with glucagon secreting alpha cells, they form the majority of the endocrine cell population of the pancreas.

The islets or islet of Langerhans are special groups of cells in the pancreas. They make and secrete hormones that help the body break down and use food. These cells sit in clusters in the pancreas. There are five types of cells in an islet: beta cells, alpha cells, delta cells, which make somatostaton, and PP cells and D1 cells.

The present invention makes use of the exocrine fraction which is normally discarded after isolation of islets of Langerhans from the pancreas of humans and other mammals. In certain embodiments pancreatic exocrine cells are derived from adult, postnatal or prenatal pancreas. In a certain embodiment, the redifferentiated endocrine pancreatic cells are used for transplantation into a different species. In another embodiment redifferentiated endocrine cells are used for transplantation into a different individual of the same species. In yet another embodiment cells, exocrine pancreatic cells are obtained from an individual and the redifferentiated endocrine cells are used for transplantation into the same individual. Dedifferentiated cells can be maintained in culture for longer periods up to 14 days. Alternatively, the dedifferentiated cells are frozen and stored.

The present invention relates to the use of EGF (epidermal growth factor) which binds to the EGF receptor (EGFR) and activates a downstream pathway. Consequently truncated or mutated versions of EGF which retain the activity of binding and activating the EGF receptor can be used as an alternative for the methods of the present invention. Within the same context, ligands such as EGF from other animal species than the species from which the pancreatic cells are isolated can be used if the bind and activate the receptor. For these purposes the sequence of such a ligand from one species can be modified in order to acquire the desired binding and activation properties on the cell population obtained from another species. For *in vivo* purposes a ligand from a non-human mammal can be "humanised" in order to acquire activity within a human and to avoid an immune response by the human immune system. Equally, other naturally or modified proteins which are a ligand for EGFR can be used for the methods of the present invention. Examples hereof are

Transforming Growth Factor-alpha, amphiregulin, betacellulin and PoxVirus Growth Factor).

The present invention relates to the use of LIF (Leukemia inhibitory factor) which binds to the gp130 receptor and activates a downstream pathway. LIF is a pleiotropic cytokine for which a function in pancreatic development has sofar not been described. It is a well-known regulator of stem cell proliferation and differentiation and is widely used to prevent differentiation of embryonic stem cells. Truncated or mutated versions of LIF which retain the activity of binding and activating the gp130 receptor can be used as an alternative for the methods of the present invention. Equally, other naturally or modified proteins which are a ligand for gp130 and activate the downstream pathway can be used for the methods of the present invention. Examples hereof are IL-6, IL-11, OSM (oncostatin M), CNTF (Ciliary Neurotrophic Factor), G-CSF (Granulocyte-colony stimulating factor), CT-1 (cardiotrophin-1) , IL-12 and Leptin.

The difference in behaviour of rodent (mouse, rat) and primate (human, monkey) cells towards LIF has been described for human ES cells and MAPC cells [Jiang et al. *cited supra*]. According to one embodiment human dedifferentiated pancreatic cells are incubated in a composition comprising a ligand for the EGF receptor (for example EGF) and a ligand for the gp130 receptor (for example LIF). In a specific embodiment wherein human cells are used, LIF is optional.

EGF, LIF and other compounds used in the methods of the present invention for the redifferentiation can be from the same species but can also be from another species as long as the compound can bind and activate its receptor. Thus in general the invention relates to the differentiation of cells of a first mammal by adding a ligand of the gp130 receptor pathway of a second mammal and/or a ligand of the EGF receptor pathway of a third mammal each of the first, second and third mammal are independently selected from the group of mammals, comprising but not limited to humans, primate and non primate monkeys, rodents such as hamster mouse and rat, rabbits, sheeps, cows and other cattle, dogs and porks.

In one embodiment the invention relates to the determination of properties in individual cells or in cell populations which are characteristic form mature insulin producing cells or characteristic for immature, dedifferentiated or embryonic cells. It was shown previously that exocrine acinar cells change their phenotype profoundly within only 4 days of suspension culture (Rooman et al. (2000) & (2001) *cited supra*). They lose amylase and other zymogens, such as pancreatic trypsinogen, trypsin and lipase, and start to express the ductal marker CK20. In Matrigel matrix they form cystic structures resembling ducts, but when they remain in suspension culture they tend to dedifferentiate and express embryonic markers like the combination of Pdx-1 and Ptf1-p48 transcription factors, the neuroendocrine marker PGP9.5, and the CCKB gastrin receptor [Rooman et al. (2000) & (2001) *cited supra*]. Apart from these markers, other markers for differentiated and dedifferentiated cells are known to the skilled person and can be used to evaluate the degree of differentiation of cells or cell populations.

The present invention shows that the redifferentiated cell populations or individual cells still have certain properties which are typical for undifferentiated or embryonic cells. Some of the redifferentiated cells which are insulin producing retain in the same cell properties such as the occurrence of two nuclei (binuclearity) and CK20 immunoreactivity. Thus, the present invention prevents markers and methods to distinguish between on the one hand redifferentiated cells and cell populations comprising redifferentiated cells and on the other hand mature cells and cell populations comprising mature cells.

In one embodiment of the present invention, dedifferentiated cells derived were cultured as monolayers attached to plastic, in the presence of low serum-concentration (1% FBS). Many alternative conditions can be envisaged for the cultivation of dedifferentiated cells of mammals, such as the use of suspension cultures, the use of serum of other animals apart from bovine serum, the use of alternative basis media other than RPMI-1640 and varying glucose concentrations.

The present invention includes a short-term culture model in which beta-cell neogenesis can be induced in certain animals such as rodents, especially

rats by the combination of two soluble factors in the medium, namely EGF and LIF. This is the first in vitro study of acinoinsular transdifferentiation, documenting the phenotypic switch from normal exocrine to endocrine cells.

One embodiment of the invention relates to a pharmaceutical composition a population of mammalian cells comprising redifferentiated insulin secreting pancreatic cells. In addition to the cell population, the composition usually includes at least a pharmaceutically acceptable carrier, well known to those skilled in the art and for instance selected from proteins such as collagen or gelatine, carbohydrates such as starch, polysaccharides, sugars (dextrose, glucose and sucrose), cellulose derivatives like sodium or calcium carboxymethylcellulose, hydroxypropyl cellulose or hydroxypropylmethyl cellulose, pregeletanized starches, pectin agar, carrageenan, clays, hydrophilic gums (acacia gum, guar gum, arabic gum and xanthan gum), alginic acid, alginates, hyaluronic acid, polyglycolic and polylactic acid, dextran, pectins, synthetic polymers such as water-soluble acrylic polymer or polyvinylpyrrolidone, proteoglycans, calcium phosphate and the like

The present invention shows the *in vitro* redifferentiation of dedifferentiated exocrine cells in the presence of compounds such as the combination of LIF. In alternative embodiments the redifferentiation of cells is envisaged to happen within the individual to be treated. As an example, dedifferentiated cells are embedded within a biodegradable matrix further comprising a compounds allowing a time and concentration controlled matrix comprising for example LIF and EGF, which allows the *in vivo* differentiation of dedifferentiated exocrine pancreatic cells. After degradation of the matrices, the differentiated cells are released. In a particular embodiment the cells are first treated with differentiating growth factors for a limited time *in vitro* and afterwards implanted in the presence of growth factors for further *in vivo* differentiation. Thus in another aspect, the invention relates to pharmaceutical composition dedifferentiated cells and compounds for the differentiation of the cells.

The following examples illustrate the present invention without being limited thereto.

Examples

Example 1: Experimental setup

Animals: Male 10-12 week old Wistar rats (Janvier, Le Genest-St-Isle, France) weighing 250-300 g were used for the isolation of cells from the pancreas. Eight-week old male BALB/cAnNCrl-*nu*BR nude mice (Charles River Laboratories,Inc. Wilmington, MA) weighing 22-24 g were used as recipients for transplantation. All animal experimentation was approved by the Ethical Committee of the Free University of Brussels.

Isolation of exocrine tissue: Pancreata were partially dissociated with collagenase and exocrine acini were purified by centrifugal elutriation as published before (Rooman et al. (2000) & (2001) *cited supra*).

Culture procedure: Exocrine cells were pre-cultured for 4 days in bacteriological petri dishes (Nunc, Naperville, Ill., USA) in suspension culture in RPMI-1640 Glutamax-I medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Paisley, Scotland), penicillin (75 mg/l) (Continental Pharma, Brussels, Belgium) and streptomycin (100 mg/l) (Sigma, St Louis, Mo., USA) and Geneticin Sulphate (50 µg/ml) (Sigma) was used to ban fibroblasts from the culture. Medium was replaced daily during this preculture period. On the fourth day after isolation cells were distributed in 1000 µl-aliquots over 24-well plates (Falcon, BD Biosciences, Erembodegem, Belgium). This procedure was standardized as to obtain approximately 75 ng DNA per well. For some experiments, the cells were treated with 10 mM alloxan for 10 minutes prior to plating. After overnight culture, nonadherent cells were washed off and then either control medium or growth factor-containing medium was added to the wells. Control medium consisted of RPMI-1640 medium supplemented with 1% FBS and antibiotics (streptomycin 0.1 g/l and penicillin 0.075 g/l). Growth factor-medium consisted of control medium supplemented with 50 ng/ml human recombinant epidermal growth factor (EGF) (Sigma, St. Louis, MO) and 40 ng/ml recombinant mouse leukemia inhibitory factor (LIF) (Sigma). Cell monolayers were analysed after a culture period of 3 days in the latter media.

Immunocytochemistry and DNA measurement: The amount of cells per well was measured by a DNA fluorimetric assay based on the binding of Hoechst

33258 dye [Loontiens *et al.* in *Biochemistry.* (1990) **29**, 9029-9039]. At least 6 wells were used per experimental condition, so that triplicate cultures could be used for DNA-extraction and, in parallel, for immunocytochemical analysis of cell composition. Immunocytochemical staining of the monolayers was performed directly in the 24-well plates. For this purpose, the cell monolayers were fixed for 10 min with 4% buffered formaldehyde followed by 20 min methanol (-20°C) for cell permeabilization. For single stainings of only one antigen, the streptavidin-biotin peroxidase method was used [Bouwens *et al.* in *Diabetes.* (1994) **43**, 1279-1283; Bouwens & De Blay in *J Histochem Cytochem.* (1996) **44**, 947-951]. For double staining, the indirect method with FITC- and TRITC-labeled secondary antibodies was used (Jackson Immunoresearch, West Grove, PA). Primary antibodies used in this study are polyclonal anti-insulin (C. Van Schravendijk, VUB, Brussels) [Bouwens *et al.* (1994) *cited supra* ; Bouwens & De Blay *cited supra*], polyclonal anti-rat C-peptide-I (O.D. Madsen, Hagedorn Research Institute, Gentofte, Denmark [Blume *et al.* in *Mol Endocrinol.* (1992) **6**, 299-307], polyclonal anti-Pdx1 (O.D. Madsen)[Rooman et al.(2000) *cited supra*], polyclonal anti-Glut-2 (Wak-Chemie, Bad Soden, Germany), monoclonal anti-cytokeratin-20 (CK20) (Novocastra, Newcastle-upon-Tyne, UK)(Bouwens *et al.* (1994) *cited supra* ; Bouwens & De Blay *cited supra*), polyclonal anti-alpha-amylase (Sigma), and mouse monoclonal anti-BrdU (ICN, Irvine, CA, USA). To assess the incorporation of 5'-bromodeoxyuridine (BrdU) by proliferating cells, 10 µM BrdU (Sigma) was added to the culture medium one hour before fixation (Rooman et al.(2001) *cited supra*). For BrdU pulse-chase labeling, BrdU was added to the culture medium on the first day of adherent cell culture and removed 24 h later. The cells were analyzed 24h after the pulse or on day 3 of cell culture, i.e. after 48h chasing in the absence of BrdU. BrdU incorporation was scored both in insulin-expressing cells and in cytokeratin-expressing cells.

RT-PCR analyses: Total RNA was extracted from monolayer cell culture of rat pancreatic cells using Trizol RNA isolation method (Invitrogen Life Technologies, Carlsbad, CA). For the semi-quantitative analysis of transcripts encoding notch-1 and glyceraldehyde phosphate dehydrogenase, the total RNA

was reversed transcribed and amplified as described by the manufacturer (Invitrogen Life Technologies). PCR products were separated by electrophoresis in 1.5-2.5% agarose gels and visualized by ethidium bromide staining. Analyses were performed at least three times.

Morphometry: Computer-assisted morphometry [Bouwens et al. (1994) cited above] was used to measure the area of monolayers in 24-well plates.

Insulin measurements: Cellular insulin content and insulin released in the medium were measured by radio-immunoassay [Pipeleers et al.*cited supra*]. To study glucose-stimulated insulin release, insulin in the culture medium was measured after a 4 hour incubation in basal medium containing 2.5 mM glucose, followed by a 4 hour incubation in 20 mM glucose (serum-and glutamine-free HAM-10 medium, Gibco) [Lobner et al. in *Diabetes* (2002) **51**, 2982-2988].

Transplantation: To transplant the monolayer cells, they were cultured on collagen S from calf skin (type I; Roche Molecular Biochemicals, Mannheim, Germany) and detached with collagenase P (Roche Molecular Biochemicals). Cell pellets were implanted under the kidney capsule (17) of nude mice that had been injected intravenously with 70 mg/kg alloxan 3 hours prior to transplantation. Blood glucose levels were monitored every two days in samples obtained from the tail vein of fed mice by using Glucocard Memory strips (A. Menarini Diagnostics Benelux, Zaventem, Belgium).

Statistics: A two-tailed, paired Student t-test was used and statistical significance was considered at a confidence interval <0.05. Mean values are given ± SEM. Repeats of independent experiments are indicated as n = x (x being the number of repeats) with each cell preparation being a pool from a total of 5 rats.

Example 2: Generation and composition of exocrine cell monolayers

Isolated and pre-cultured exocrine acinar cell aggregates were obtained as described earlier (Rooman et al.(2000) & (2001) *cited supra*) and subsequently allowed these cells to form monolayers on plastic. More than 90% of the cells were immunoreactive for the ductal marker CK20 and had lost the

amylase marker of acinar cells. (Of these cells, 79.0 ± 0.4% (n=7) were binucleated, a characteristic of part of the acinar cells. These cultures were initially contaminated with 3.7 ± 0.46 % (n=7) insulin-positive cells. When the cell preparations had been pre-treated with alloxan prior to monolayer formation, the monolayers contained less than 0.5% insulin-positive cells. When geniticine was used during the cultivation, the outgrowth of fibriblastic cells was inhibited.

Published data showed that exocrine acinar cells lose their differentiated characteristics like amylase and other zymogens within 4 days of suspension culture and start to express ductal and embryonic characteristics like CK20, the combination of Pdx-1 and Ptf1-p48 transcription factors, the neuroendocrine markers PGP9.5 and the CCKB gastrin receptor (Rooman et al. (2000) & (2001) *cited supra*; Lardon et al.(2004) *Virchows Arch.* **444**, 61-65). In the presence of dexamethasone, it was shown that these cells can transdifferentiate into hepatocyte-like cells [Lardon et al. (2004) in Hepatology 39, 1499-1507]. For the present study these cells were cultured as monolayers attached to plastic, in the presence of low serum-concentration (1% FBS) and were tested for the effect of the combination of LIF and EGF. These factors are known to control the differentiation of adult and embryonic neural stem cells (Viti et al. (2003) in *J. Neurosci.* **15**, 3385-3393).

Example 3: Effect of growth factors on cell number and beta-cell frequency

When monolayer cultures were treated with LIF and EGF for 3 days, a significant increase in DNA content was noted compared to the start of the culture and compared to cultures devoid of both growth factors (Fig. 1A). The total surface covered with cells which was measured by morphometry showed a comparable increase.

Treatment with LIF and EGF also induced a significant increase in the frequency of insulin-positive cells, expressed as the percentage of all cells (Fig. 1B). When multiplying the frequency of insulin-positive cells with total DNA-content to have a measure of the absolute beta-cell number, an 11-fold

increase in beta-cell number was observed in the presence of LIF and EGF over the 3-day culture period (Fig. 1C). Analysis of total cellular insulin content by radio-immunoassay showed a 6-fold increase in LIF and EGF treated cultures compared to control cultures(Fig. 1D).

In the presence of either LIF or EGF alone there was also a significant increase in beta-cell number, respectively 6- and 5-fold compared to the start material, but these increases were significantly lower than in the presence of both factors together.

In alloxan pre-treated cultures depleted of contaminating beta-cells, and after 3 days of culture in the presence of LIF and EGF, 9.2 ± 1.5% insulin-positive cells were found (n = 4). In the absence of growth factors there were 0.4 ± 0.1% insulin-positive cells. The upregulation of insulin-positive cells which was seen with the combination of both factors, was not observed when either EGF or LIF was given alone.

The cells populations which are obtained according to the methods of the present invention can be further enriched for insulin secreting cells by methods such as FACS sorting, size fractionation and elutriation as explained in e.g. EP1146117.

In vitro, EGF or LIF alone also had an effect on the number of beta cells. This can be explained by the observation that these factors are also produced by the cultured cells and can work in a paracrine or autocrine manner (unpublished observations). In the present invention it was shown that LIF is produced by beta cells, thus suggesting that beta cells can stimulate their own regeneration from the precursor cells. Beta cells also produce other factors which can regulate islet neogenesis, for example netrin [De Breuck et al. in *Diabetologia* (2003) **46**, 926-933] and vascular endothelial growth factor [Rooman et al. in *Lab Invest.* **76**, 225-232]. These observations may explain why "spontaneous" islet regeneration following toxic or autoimmune insult is limited.

In the present model of dedifferentiated exocrine cells, the combination of LIF and EGF was found to stimulate cell proliferation as evidenced by a significant increase in total DNA and a larger area occupied by the monolayers. The combination of LIF and EGF also resulted in an approximate sixfold

increase in the percentage of insulin-positive cells. Combined with the observed increase in DNA, this gives more than a tenfold increase in number of insulin-containing cells.

Example 4: Phenotypic analysis

Analysis by double immunofluorescence staining showed that most of these insulin-positive cells also expressed other beta-cell markers, including proinsulin C-peptide-I, the transcription factor Pdx-1, and the glucose transporter Glut-2. Immunoreactivity for C-peptide excludes the possibility of insulin uptake from the medium as an explanation for the increased frequency of insulin-immunoreactive cells (insulin was not added to the medium).

In the EGF+LIF-treated cultures, approximately 10% of the insulin-positive cells were immunoreactive for CK20, with a somewhat lower staining intensity compared to insulin-negative cells. Co-expression of insulin and CK20 was rarely observed in control cultures where no growth factors were added and it was not seen in the starting material. This co-expression suggests a phenotypic transition from exocrine to insulin-positive cells. Strikingly, insulin and CK20-double-positive cells were observed that were binuclear. Binuclear insulin-positive cells were rarely seen in control cultures and were absent at the start, but they were frequently seen in EGF+LIF-treated cultures where they accounted for 6.2 ± 0.47 % (n= 7) of all insulin-positive cells. In exocrine cell-derived monolayers of the present invention, nearly 80% of the cells were binuclear. Since binuclearity is a characteristic of the majority of acinar exocrine cells [Ramiya et al.*cited supra*], these observations are another indication for a transition from exocrine cells to insulin-positive cells. The redifferentiated cells which were obtained using the method of the present invention have the striking characteristic that the population as a whole, but also some individual cells still retain both the features of fully differentiated cells but also of incompletely differentiated embryonic or adult precursor cells. These features are exemplified in this and the following examples. This property of harbouring both markers of differentiated and undifferentiated cells allows the distinction between cells

obtained by the present method and freshly isolated cells from a pancreas, especially from a pancreas of an adult.

The majority of these insulin-containing cells could be considered as mature beta-cells since they expressed other phenotypic characteristics of beta-cells, such as the insulin-transactivating transcription factor Pdx-1, the beta-cell specific glucose transporter Glut-2, and the C-peptide-I component of unprocessed proinsulin. There were very few BrdU-labeled beta-cells so that the observed increase in their number within a period of 3 days must be attributed to the differentiation of precursor cells, or neogenesis.

There were two indications that the exocrine cells served as precursor cells of the newly formed beta-cells. First, CK20 immunoreactivity was noted in part of the insulin-positive cells. It was shown before that beta-cells containing CK20 are only found in the fetal [Bouwens & DeBlay, *cited supra*] and neonatal pancreas [Bouwens et al.(1994), *cited supra*], and in adult pancreas when neogenesis has been induced by duct-ligation [Wang et al.*cited supra*]. Thus, CK20 expression is a good indication for a transition from the CK20-positive exocrine cells to beta-cells. Second, binuclearity was noted in part of the insulin-positive cells. Binuclearity is a characteristic of acinar exocrine cells, and is not observed in normal rat beta-cells [Rooman et al.(2000) *cited supra*]. This is another indication for a transition from the exocrine cells to beta-cells. It is unlikely that binuclear beta-cells would represent dividing cells blocked before cytokinesis since there was very little beta-cell mitotic activity (BrdU-incorporation). Cell fusion between beta-cells can not be completely excluded although this would rather lead to a decrease in beta-cell number instead of the observed increase. Unpublished observations with mitomycin-c and cytochalasin-B predict that insulin-positive cells could also arise from binucleated (exocrine) cells by a kind of delayed cytokinesis or cleavage process. Somatic cell cleavage or reduction-division has been demonstrated indirectly from tetraploid hybrid cells but the mechanism has not yet been characterized [Wang et al. in *Nature* (2003) **422**, 897-901].

Example 5 : Proliferation

Analysis of BrdU-incorporation in insulin-positive cells showed that less than 0.1% of insulin-positive cells were BrdU-labelled after a pulse of one hour. This makes it highly unlikely that binuclearity in these cells would have resulted from arrested nuclear division in mitotic beta-cells, or that the increase in beta-cell number would have resulted from proliferation of contaminating beta-cells. Approximately 1.5% of the exocrine cells were BrdU-labeled.

A BrdU pulse-chase experiment over a 72-hour period (a pulse period of 24 hours and a chase of 48 hours) revealed a significant increase in BrdU-labeled beta-cells in the presence of EGF and LIF during the chase period (Fig. 2B). This increase can only be explained by the differentiation of proliferating exocrine cells into non-proliferating beta-cells.

Data with mitomycin-C (inhibitor of proliferation) and cytochalasin-B (inhibitor of cytokinesis) indicate that binucleated cells were able to divide into mononucleated cells (expressing insulin) without going through the cell cycle, suggesting the existence of a kind of delayed cytokinesis or cleavage phenomenon.

Example 6 : PCR analysis of mature and embryonic markers

RT-PCR analysis revealed that Notch expression was absent in the monolayers cultured in control-medium (day 8) and that Notch was upregulated in the monolayers in the presence of EGF and LIF. Notch is absent in rat islets.

Example 7: Insulin secretion

Insulin secretion studies were done over 4 h in the above mentioned cell culture conditions. Compared to their basal secretion in 2.5 mM glucose, stimulation by 20 mM glucose gave a 4-fold increase in secreted insulin, namely from 2.3 to 9.5 ng/ml.

Example 8: Transplantation

Detached monolayers of dedifferentiated pancretic rat cells treated with LIF and EGF were transplanted and observed into alloxan-treated nude mice.

Mice treated with 70 mg/kg alloxan remain permanently hyperglycemic or die within one week [Wang *et al.* in *Diabetologia.* (1995) **38**, 1405-1411]. Engraftment resulted in normalization of blood glycemia which was retained over a 14 day period but promptly reverted to hyperglycemia when the graft-bearing kidney was removed (Figure 3). This demonstrates that the redifferentiated beta cells obtained in vitro with LIF and EGF, in accordance with the present invention are able to control blood glycemia *in vivo*.

Claims

1. An *in vitro* method of generating insulin producing beta cells from a population comprising dedifferentiated exocrine pancreatic cells of a first mammal, said method comprising the steps of:
   -a) providing a population comprising dedifferentiated exocrine pancreatic cells in a culture medium
   -b) adding one or more ligands of the gp130 receptor of a second mammal and/or adding one or more ligands of the EGF receptor of a third mammal to said culture medium,
   -c) incubating said dedifferentiated exocrine pancreatic cells in said culture medium comprising said one or more ligands of the gp130 receptor and/or said one or more ligands of the EGF receptor.

2. The method according to claim 1 wherein said ligand of said gp130 receptor is a human or humanised ligand of said gp130 receptor.

3. The method according to claim 3 or 2, wherein said ligand of said gp130 receptor is LIF.

4. The method according to any of claims 1 to 3, wherein the ligand of said gp130 receptor is human or humanised LIF.

5. The method according to claim 3 or 4, wherein LIF is added to said culture medium in a concentration between 10 and 100 ng/ml.

6. The method according to claim 1, wherein said ligand of said EGF receptor is a human or humanised ligand of said EGF receptor.

7. The method according to claim 1 or 6, wherein said ligand of said EGF receptor is EGF.

8. The method according to claim 6 or 7, wherein said ligand of said EGF receptor is human or humanised EGF.

9. The method according to any of claims 6 to 8, wherein EGF is added to said culture medium in a concentration between 10 and 100 ng/ml.

10. The method according to any of claims 1 to 9, wherein the method further comprises the step of adding bFGF to said culture medium during step b).

11. The method according to any of claims 1 to 10, wherein in step b) one or more of said ligands of the gp130 receptor and/or one or more of said ligands of said EGF receptor are added to said culture medium in a concentration between 1 and 10 000 ng/ml.

12. The method according to any of claims 1 to 11, wherein said medium is free from KGF or a gastrin/CCK receptor ligand.

13. The method according to any of claims 1 to 12, wherein said incubation step is performed during less than 5 days.

14. The method according to any of claims 1 to 13, wherein the population comprising dedifferentiated exocrine pancreatic cells is selected from the group consisting of duct cells, acinar cells and islet cells.

15. The method according to any of claims 1 to 14, further comprising, prior to step a), a preliminary step of depleting said population from beta cells.

16. The method according to any of claims 1 to 15, wherein the mammalian cells are human cells.

17. The method according to any of claims 1 to 16, wherein the mammalian cells are rat cells, wherein one or more ligands of said EGF receptor

comprise human EGF and/or wherein said one or more ligands of gp130 receptor comprise murine LIF.

18. A population of mammalian pancreatic cells comprising mammalian insulin producing beta cells obtainable by a method according to any of claims 1 to 17.

19. The population of mammalian pancreatic cells according to claim 18, wherein said population comprises from about 5 to about 15 percent of insulin-positive cells.

20. A population of mammalian pancreatic cells according to claim 18 or 19, wherein said cell population after exposure to 20 mM glucose for 4 hours at 37 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum shows a more than 2 fold increase in insulin secretion when compared to the insulin secretion prior to said exposure to glucose.

21. A population of mammalian pancreatic cells according to claim 18 or 19, being able to provide an insulin secretion of at least 10 ng/ml after exposure of said population to 20 mM glucose for 4 hours at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine.

22. A population of mammalian cells comprising mammalian insulin producing beta cells, wherein said cell population comprises cells having at least one feature of a differentiated beta cell and at least one feature of an undifferentiated beta cell in the same individual cell.

23. The population of mammalian pancreatic cells according to claim 22, wherein a feature of a differentiated beta cell is insulin secretion and wherein a feature of an undifferentiated beta cell is CK20 expression and/or binuclearity.

24. A population of mammalian pancreatic cells according to claim 22 or 23, obtainable by the method of any of claims 1 to 18.

25. A population of mammalian pancreatic cells comprising mammalian insulin secreting beta cells wherein said cell population comprises a first subpopulation of cells having markers of undifferentiated or dedifferentiated cells and comprises a second subpopulation of cells having markers of differentiated cells.

26. The population of mammalian pancreatic cells according to claim 25, wherein the markers of differentiated cells are selected from the group of consisting of C-peptide-I, Pdx-1, Glut-2 and insulin.

27. The population of mammalian pancreatic cells according to claim 26, wherein the markers of dedifferentiated or undifferentiated cells are selected from the group of cytokeratin 7, cytokeratin 19, cytokeratin 20, CCKB receptor for gastrin, PGP9.5 and notch-1 receptor.

28. A population of mammalian pancreatic cells according to any of claims 18 to 27, being obtainable by the method of any of claims 1 to 17.

29. The population of mammalian pancreatic cells according to any of claims 18 to 28, wherein said mammalian cells are human cells.

30. The population of mammalian pancreatic cells according to any of claims 18 to 29, wherein said mammalian cells are porcine cells.

31. The population of mammalian pancreatic cells according to any of claims 18 to 30, wherein said mammalian cells are rat cells.

32. A pharmaceutical composition comprising a therapeutically active amount of a mammalian pancreatic cell population according to any of claims 18 to 31, and at least one pharmaceutically acceptable carrier.

33. Use of a mammalian pancreatic cell population according to any of claims 18 to 31, for the manufacture of a medicament.

34. Use according to claim 33, wherein the medicament is used for the treatment of diabetes type 1 or type 2.

35. A method for the treatment of diabetes type 1 or type 2 comprising the step of administering an effective amount of the pharmaceutical composition of claim 32 to an individual in need of it.

36. Use of a combination of a human or humanised ligand of a EGF receptor, and a human or humanised ligand of the gp130 receptor for the preparation of a medicament.

37. Use according to claim 36, wherein said medicament is used for the treatment of diabetes type 1 or type 2.

38. Use according to claim 36 or 37, wherein the human or humanised ligand of a EGF receptor is human EGF and the human or humanised ligand of the human gp130 receptor is human LIF.

39. Use of a human or humanised ligand of the gp130 receptor for the preparation of a medicament for the treatment of diabetes type 1 or type 2.

40. Use according to claim 39, wherein said human or humanised ligand of the gp130 receptor is LIF.

41. An *in vitro* method for determining the degree of redifferentiation of dedifferentiated mammalian pancreatic cells comprising the steps of determining one or more parameters selected from the group consisting of:
a) The presence of CK20, CK7 or CK 19
b) the ocurrence of binucleated cells
c) the presence of insulin positive cells
d) the presence of C-peptide, Pdx-1 and Glut-2
e) the presence of gastrin CCKB receptor, PGP9.5 and notch-1 receptor on said mammalian pancreatic cells.

42. A population of mammalian pancreatic cells according to any of claim 18 to 31, being identifiably by the method of claim 41.

43. A method of generating *in vitro* insulin producing mammalian beta cells from dedifferentiated pancreatic cells comprising the step of:
-incubating said dedifferentiated pancreatic cells in a medium comprising a ligand of the gp130 receptor.

44. The method according to claim 44 wherein the medium further comprises a ligand of the EGF receptor.

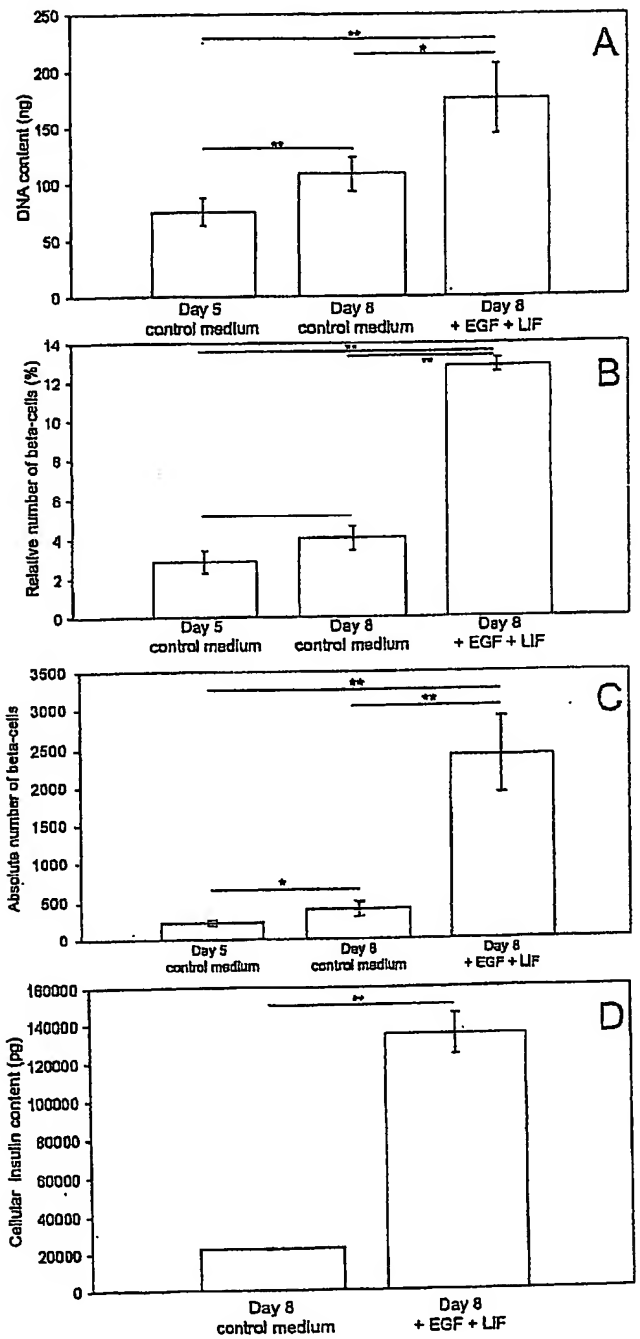
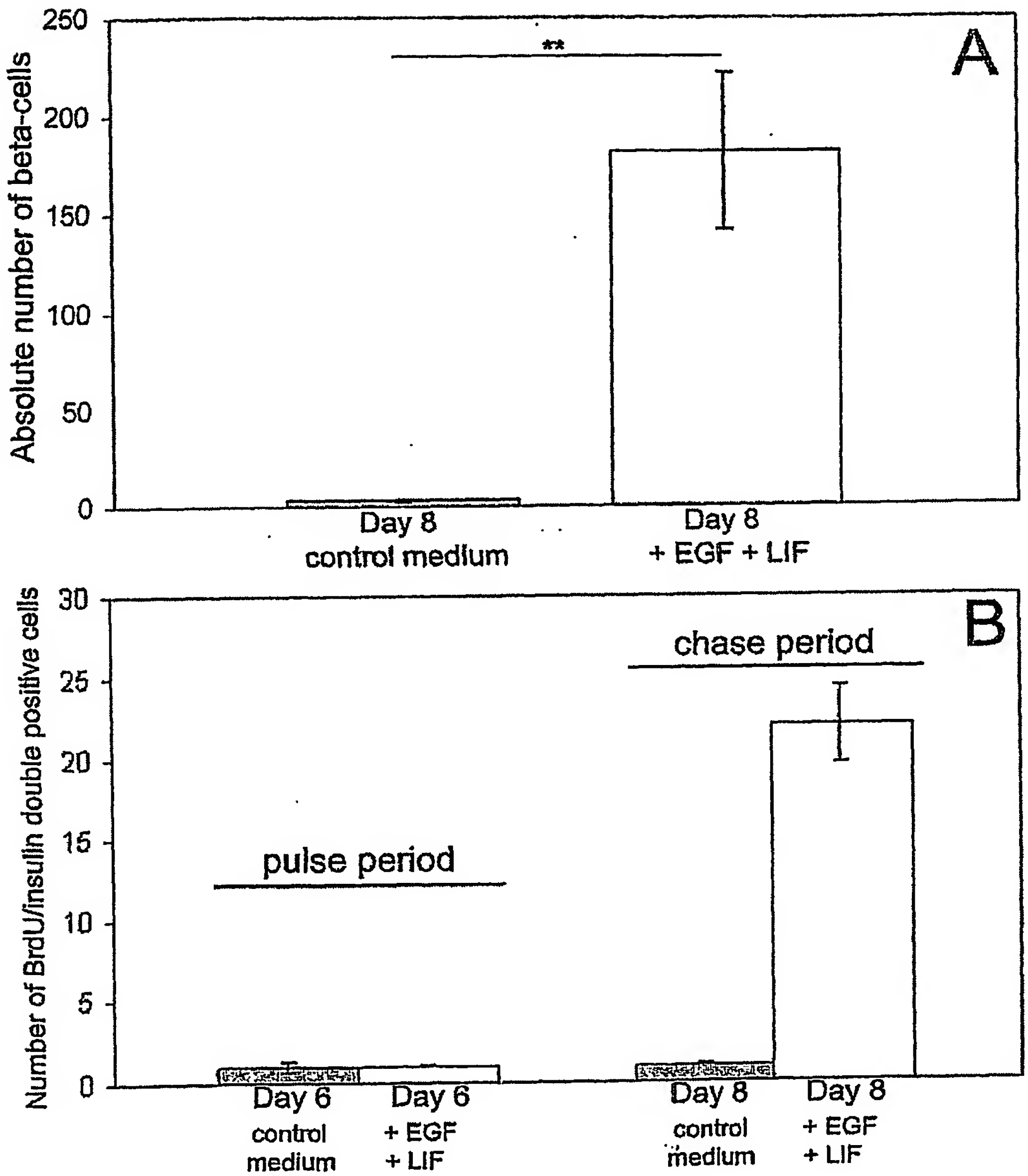
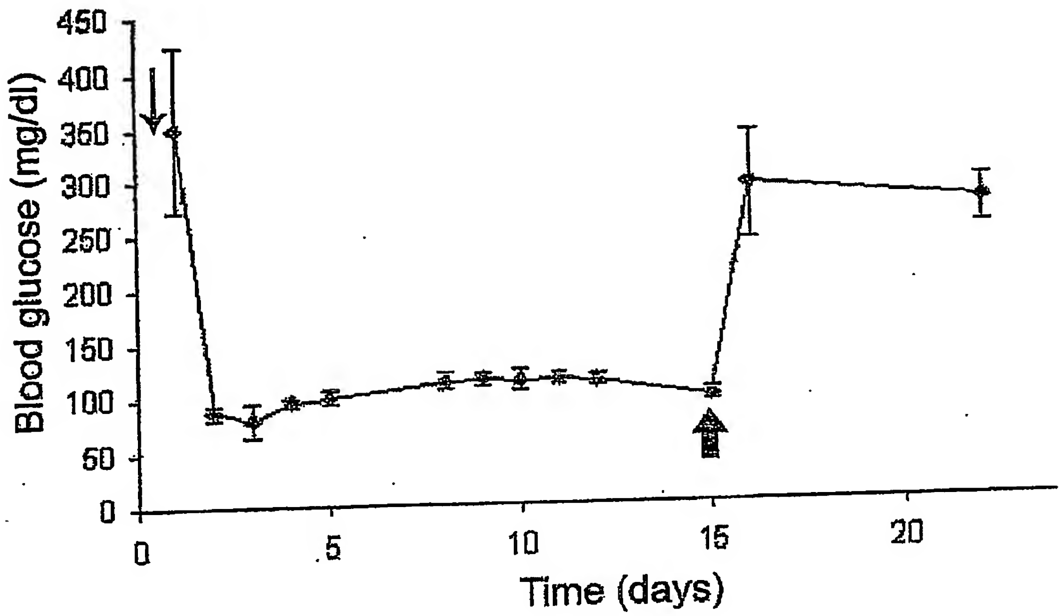

A
DNA content (ng)
250
200
150
100
50
0
Day 5
control medium
Day 8
control medium
Day 8
+ EGF + LIF
B
Relative number of beta-cells (%)
14
12
10
8
6
4
2
0
C
Absolute number of beta-cells
3500
3000
2500
2000
1500
1000
500
D
Cellular insulin content (pg)
160000
140000
120000
100000
80000
60000
40000
20000

Figure 1



250
200
150
100
50
0
Absolute number of beta-cells
**
A
Day 8
control medium
Day 8
+ EGF + LIF
30
25
20
15
10
5
0
Number of BrdU/insulin double positive cells
B
pulse period
chase period
Day 6
control
medium
Day 6
+ EGF
+ LIF
Day 8
control
medium
Day 8
+ EGF
+ LIF

Figure 2



450
400
350
300
250
200
150
100
50
0
Blood glucose (mg/dl)
0
5
10
15
20
Time (days)

Figure 3